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Chondrocyte primary cilia shorten in response to osmotic challenge and are sites for endocytosis

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SUMMARY

Objective: The purpose of this study was to examine the influence of cartilage site and osmolarity on primary cilia incidence, length and orientation in live chondrocytes in undisturbed cartilage. Additionally, we imaged endocytotic markers to test our hypothesis that the ciliary pocket is a site for endocytosis.

Materials and methods: We measured primary cilia incidence, length and orientation in the coronal plane using *ex vivo* live cell confocal imaging of intact murine femoral chondrocytes. Measurements were taken from five regions of the medial and lateral condyles of the left and right femur and also after one minute of osmotic challenge. Transmission electron microscopy and immunocytochemistry were used to characterize the orientation and position of chondrocyte primary cilia in the saggital plane and to determine the colocalization of clathrin coated vesicles, endosomal and lysosomal proteins and CD44 with the ciliary pocket.

Results: Chondrocyte primary cilia length decreased significantly after a one minute hypo- or hyper-osmotic challenge and varied between condyles and across the surface of each condyle. The majority of the length of the chondrocyte primary cilia was positioned within a membranous invagination rather than projecting out from the cell membrane and clathrin coated vesicles, endosomal proteins and CD44 colocalised with the ciliary pocket.

Conclusions: We demonstrate that live *ex vivo* chondrocyte primary cilia are capable of shortening within minutes in response to osmotic challenge and provide subcellular and cellular evidence that chondrocyte primary cilia are deeply invaginated in a ciliary pocket which contains sites for endocytosis.

Introduction

Primary cilia have been identified in almost all vertebrate cells[1,2]. They are immotile consisting of a 9 + 0 arrangement of microtubules, lacking the inner doublet and corresponding molecular motors necessary for motility. Microtubules themselves are polymers of α and β tubulin with both a (+) and a (−) end[3,4]. Microtubules elongate and resorb from the (+) end and therefore primary cilia shortening occurs from the distal tip in a complex process involving intraflagellar transport[3,4].

Electron microscopy studies have shown that primary cilia often reside in invaginations of the plasma membrane called the ciliary pocket[5–9]. In early work, clathrin coated pits and vesicles were frequently reported along the membrane of the ciliary pocket and at the ciliary base suggesting a potential involvement in receptor mediated endocytosis[5,6]. Most recently it has been shown that an actin-cilium interface is able to modulate the local environment of the ciliary pocket[7,8]. The ciliary pocket-primary cilia complex is a site of endocytosis as well as primary cilia based signal transduction pathways in synoviocytes[8,10].

In addition to their role in endocytosis, primary cilia may play a critical role in signal transduction. Numerous cell signalling and cell adhesion proteins have been found on chondrocyte primary cilia including the integrin subunits α2, α3, and β1[11], the mechano-sensitive ATP release channel, Connexin 43[12] and the osmotically sensitive cation channel transient receptor potential vanilloid 4 (TRPV4)[13]. Together, these findings indicate that the chondrocyte is able to respond to changes in the extracellular matrix by utilizing proteins and signalling molecules associated with the primary cilium.

Most recently it has been shown that compression produces a reversible reduction of both primary cilia length and incidence in chondrocytes[14]. The presence of TRPV4 along the length of the primary cilia[13] together with the potentially heightened sensitivity

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of the cilium to fluctuations in osmotic pressure due to its 4,000 fold smaller volume relative to the cell body[5] may suggest that primary cilia are involved in the sensing and transduction of osmotic as well as mechanical challenges to chondrocytes. Chondrocytes experience fluctuations in their osmotic environment during cartilage compression and relaxation as water is extruded and then imbibed into the negatively charged proteoglycan rich extracellular matrix[15,16]. Additionally, during osteoarthritis the cartilage matrix loses proteoglycan molecules and the surface integrity is compromised leading to tissue swelling and a chronic decrease in interstitial osmolarity[17]. It is currently unknown if the incidence and length of chondrocyte primary cilia are modulated by osmotic challenge.

Chondrocyte primary cilia incidence and length have been reported extensively in the literature and have been shown to vary greatly with species, cartilage surface, and tissue preparation (explants, agarose constructs or culture). Early studies on equine and murine chondrocyte primary cilia of the femoral condyle found that virtually all chondrocytes have at least one cilium[18]. Later studies on bovine patella explants found a primary cilia incidence of 46%[19] and reported primary cilia lengths ranging from 1.1 μm in the superficial layer of cartilage to 1.5 μm in the deep layer[19]. Compared to chondrocytes in explants, chondrocytes in culture tend to have longer primary cilia (1.3–2.2 μm) but a lower primary cilia incidence (26–28%)[14,20].

The disparity between *in situ* and *in vitro* measurements of chondrocyte primary cilia incidence and length underlines the importance of the extracellular matrix in influencing these attributes. The explant studies in the literature have machined the subchondral bone[18,19] perhaps disrupting the anchoring of the collagen fibrillar network into the bone[21] and thus altering the pre-stressed conditions experienced by the chondrocytes in the proteoglycan rich matrix[22]. These properties of the extracellular matrix and the interconnectedness of the primary cilia with it *via* integrin molecules[11], necessitate the utilization of whole cartilage-bone constructs in these studies. To date chondrocyte primary cilia incidence and length have not been measured in fully intact and undisturbed cartilage.

In addition to sample preparation, the animal species and/or the specific joint surface from which the cartilage was harvested may influence chondrocyte primary cilia. It is well established that cartilage and chondrocyte properties vary significantly between different joints of the same body and even across different surfaces of the same joint. For example, even under identical load magnitudes the cartilages of the patellofermoal joint differ in histological, material and compositional properties and their chondrocytes in anabolic and catabolic metabolism[23]. In addition, cartilage thickness and collagen fibre split line orientation have been shown to vary significantly across the articulating surfaces of the femur[24]. To date, the influence of site across the same cartilage surface on primary cilia incidence, length and orientation has not been measured.

The purpose of this study was to characterize primary cilia in femoral chondrocytes *in situ* using transmission electron microscopy and immunocytochemistry and *ex vivo* using live cell confocal imaging. For the first time, we examine the influence of cartilage site and osmolarity on primary cilia incidence, length and orientation in live chondrocytes on the condyles of intact murine femora. We hypothesised that primary cilia incidence and length would be greater on the more central, constantly loaded regions of the cartilage compared to peripheral intermittently loaded regions and that changes in osmolarity would also influence primary cilia incidence and length. Additionally, we imaged endocytotic markers at the cellular and subcellular level to test our hypothesis that the ciliary pocket is a site for endocytosis.

Materials and methods

Animals

All animal procedures were approved by the University of Calgary Animal Care Committee. Skeletally mature (age = 4.0 ± 0.5 months, mass = 26 ± 12 g (mean ± sd)) female Balb/C mice were euthanized and the femora were isolated and placed in fixative for electron microscopy and immunocytochemistry or iso-osmotic (300mOsm) Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Invitrogen, Burlington, ON) for live cell confocal imaging.

Transmission electron microscopy

Femora were fixed in 3% glutaraldehyde in Millonigs buffer (300mOsm) at room temperature overnight prior to post fixation in 2% osmium tetroxide for 2 hours. Samples were dehydrated in ethanol and then infiltrated with Polybed 812 resin (Polysciences, Warrington, PA). Polymerization was performed at 37°C for 24 hours. Silver grey sections were cut with a diamond knife on an ultra microtome (Leica, Concorde, ON). Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H7650 electron microscope.

Immunocytochemistry

Femora were fixed in 4% paraformaldehyde, decalcified (CalEX, Fisher Scientific, Ottawa, ON) and then submerged in 25% sucrose overnight. Samples were embedded in optimal cutting temperature compound and flash frozen. Sagittal sections (12 μm) were cut, washed (phosphate buffered saline (PBS)) and blocked (normal goat serum and Triton X (Sigma, Oakville, ON)) before going through sequential wash (PBS and Tween 20 (Sigma, Oakville, ON)) and antibody application steps. Primary antibodies included acetylated α-tubulin (clone 6-11B-1, 1:500, Sigma, Oakville, ON), early endosome antigen-1 (EEA-1) (1:200, Abcam, Cambridge, MA), Lysosomal-associated membrane protein 1 (LAMP-1-FITC conjugated) ([1D4B], 1:10, Abcam, Cambridge, MA) and CD44 (1:10, Abcam, Cambridge, MA). Secondary antibodies included Alexa Fluor® 488 and Alexa Fluor® 647 (Invitrogen, Burlington, ON). Finally, the nucleic acid stain Hoescht 33342 (0.1 mM, Invitrogen, Burlington, ON) was applied to all sections. After staining, sections were mounted in ProLong® Gold (Invitrogen, Burlington, ON) and coverslipped.

Slides were imaged using an oil immersion lens (40×, 1.4 N.A.) on an LSM 7 DUO (Carl Zeiss Canada Ltd., Toronto, ON) confocal microscope in either differential interference contrast (DIC), channel or Lambda scanning confocal modes. In the channel scanning confocal mode dyes were sequentially scanned; Hoescht (excitation 405 nm, emission 429–684 nm) and Alexa Fluor® 647 (excitation 633 nm, emission 638–755 nm). At least 30 cells located in each of the superficial zone, middle zone, calcified cartilage region or within a chondron were identified. In Lambda scanning confocal mode, sections stained with either Hoescht, Alexa Fluor® 488 or Alexa Fluor® 647 were excited and the resulting emission spectra recorded across 8.8 nm bands from 411–692 nm. An additional autofluorescence curve was recorded from an unstained section simultaneously excited at 405 nm, 488 nm and 633 nm. On a triple stained section simultaneously excited at all three wavelengths a cell of interest was scanned and the resulting emission spectra linearly unmixed using the prerecorded emission curves.

Live cell confocal imaging

Chondrocytes of the intact femora were incubated with 125 nM Tubulin Tracker (Invitrogen, Burlington, ON) to stain polymerized

tubulin followed by 4 μM Ethidium homodimer-1 (Invitrogen, Burlington, ON) to stain the nuclei of dead cells. Femora were washed before being secured (condyles facing up) inside a heated perfusion chamber (Carl Zeiss Canada Ltd., Toronto, ON) and submerged in 300mOsm DMEM. The perfusion chamber was kept at 37 ± 0.5°C throughout experimentation.

Confocal microscopy (Olympus Fluoview FV 1000, Tokyo, Japan), excitation 488 nm, emission 500–600 nm and 630–780 nm, was utilized to image the primary cilia of live chondrocytes in fully intact murine femora. Images were taken as Z stacks with either a 40×/0.8N.A. (osmotic experiments, optical slice thickness 2.568 μm) or 60×/0.9N.A. (cartilage site experiments, optical slice thickness 1.99 μm) dipping objective (Olympus, Tokyo, Japan). For the cartilage site experiments images were taken in the centre of the condyle, and one field of view (210 μm or 300 μm) towards the edges of the condyle (top, bottom, inside, and outside) thus covering the entire cartilage surface of the murine condyle (<1 mm diameter) (Fig. 1). For the osmotic experiments, 300mOsm DMEM was withdrawn from the chamber and 200, 300, or 400mOsm DMEM was perfused. Osmolarity was manipulated by the addition of either distilled water or sucrose. Z stacks were taken in the centre and top regions one minute after the medium change.

Z stacks containing at least 20 living cells were selected for analysis. Minimally, sixty cells from each region were measured. Primary cilia length was measured with an electronic ruler using the optical slice with the longest ciliary profile for each cell (FV10-ASW 2.0 Viewer software, Olympus, Tokyo, Japan). To measure primary cilia orientation in the coronal plane, a cross was digitally placed in the centre of each cell to divide the cell into quadrants. The quadrant where the primary cilia was located was noted. Two measurements were made on each cell, one with the cross placed to look like a '+' and the second with the cross placed at 45 degrees to look like a 'x'.

Variation of primary cilia incidence, length and orientation with femora (left/right), condyle (medial/lateral), and region (centre, top, bottom, inside, and outside) were investigated. Multivariate analysis of variance (ANOVA) with Fisher least significant difference (LSD) *post hoc* tests (primary cilia length data) and chi squared tests (non parametric primary cilia incidence and orientation data) were performed using Statistica software (Statsoft, Tulsa, OK).

Results

Position and orientation of chondrocyte primary cilia

To examine the primary cilia on murine femoral chondrocytes on a subcellular level transmission electron microscopy was utilised. Chondrocyte primary cilia were positioned within membranous invaginations into the cell and close to the nucleus, rather than projecting out from the cell membrane [Fig. 2(A–C)]. Within the cytoplasm, the primary cilia was surrounded by a ciliary pocket where endocytotic vesicles were located [Fig. 2(B–D)].

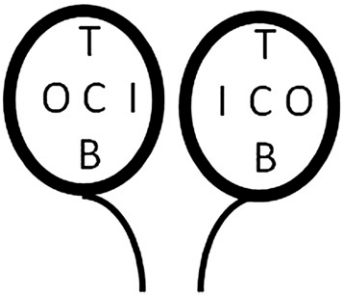

Fig. 1. Diagram showing the different areas imaged on the intact murine femoral condyles for live cell microscopy. Images were taken in the centre (C) of the condyle and one field of view towards the edges - top (T), outside (O), inside (I) and bottom (B).

To evaluate the position and orientation of chondrocyte primary cilia in the sagittal plane immunocytochemistry was employed. Primary cilia were imaged as areas of highly concentrated acetylated α-tubulin adjacent to the nucleus (Fig. 3). In agreement with the transmission electron microscopy, the immunocytochemistry depicts the primary cilia shaft largely invaginated in a membranous pocket within the chondrocyte close to the nucleus and not protruding into the extracellular matrix [Fig. 3(A,C,E)]. Throughout the depth of cartilage tissue, single primary cilia are located on the basal side of the chondrocytes away from the articular surface [Fig. 3(A–D)]. Chondrocytes partly embedded in the calcified cartilage region have primary cilia that are more randomly oriented, often on either of the two sides perpendicular to the cartilage/bone interface [Fig. 3(E–F)]. Finally, chondrocytes located with other cells within a common chondron have primary cilia located on the side nearest to the other chondrocyte(s) in the chondron [Fig. 3(G–H)].

To measure the position and orientation of primary cilia on live chondrocytes in the coronal plane polymerized tubulin in intact murine femora was visualised. Due to the high concentration of tubulin in the primary cilia, the primary cilia emit greater fluorescence intensity relative to the rest of the cell cytoskeleton (Fig. 4). In agreement with the electron microscopy and immunocytochemistry, chondrocyte primary cilia were seen to have one end close to the nucleus (darker circles) and the other end close to the extracellular matrix with the ciliary shaft extending within a membranous invagination through the cytoplasm (Fig. 4). Surprisingly, there was no statistically significant relationship between chondrocyte primary cilia orientation and femora (left/right), condyle (medial/lateral), region (centre, top, bottom, inside, and outside) or osmotic challenge (200mOsm/300mOsm/400mOsm) (data not shown).

Effects of cartilage site and osmolarity on primary cilia incidence and length

To investigate the effects of cartilage site and osmolarity on primary cilia incidence and length we imaged the polymerized tubulin of live chondrocytes in intact murine femora. Single primary cilia were present on 87% of chondrocytes and no chondrocyte had more than one primary cilia. The percentage of chondrocytes with primary cilia was greater on right femora (89%) than on left femora (85%) ($P = 0.03$). The variation of primary cilia incidence with condyle (medial/lateral), cartilage region (top/bottom/center/inside/outside) or osmolarity (200mOsm/300mOsm/400mOsm) was not statistically significant (data not shown).

The average length of primary cilia across the femoral condyles was 0.81 ± 0.24 μm (mean ± sd). Primary cilia length varied between condyles and across the surface of each condyle. Primary cilia in the inside and outside regions were longer on the medial than the lateral condyle ($P < 0.001$, $P = 0.002$ respectively) [Fig. 5(a)]. On chondrocytes of the medial condyle, primary cilia were longest in the inside region ($P = 0.019$) however on the lateral condyle primary cilia tended to be longest in the central region [Fig. 5(a)]. Following application of 200mOsm and 400mOsm media for one minute, primary cilia length was significantly shorter compared to chondrocytes in 300mOsm control media ($P = 0.002$, $P = 0.045$ respectively) [Fig. 5(b)]. No significant difference in primary cilia length was observed between the 200mOsm and 400mOsm treatments themselves ($P > 0.05$) [Fig. 5(b)].

Primary cilia and endocytosis

To investigate the ciliary pocket as a site for chondrocyte endocytosis we performed immunocytochemistry to visualise EEA-

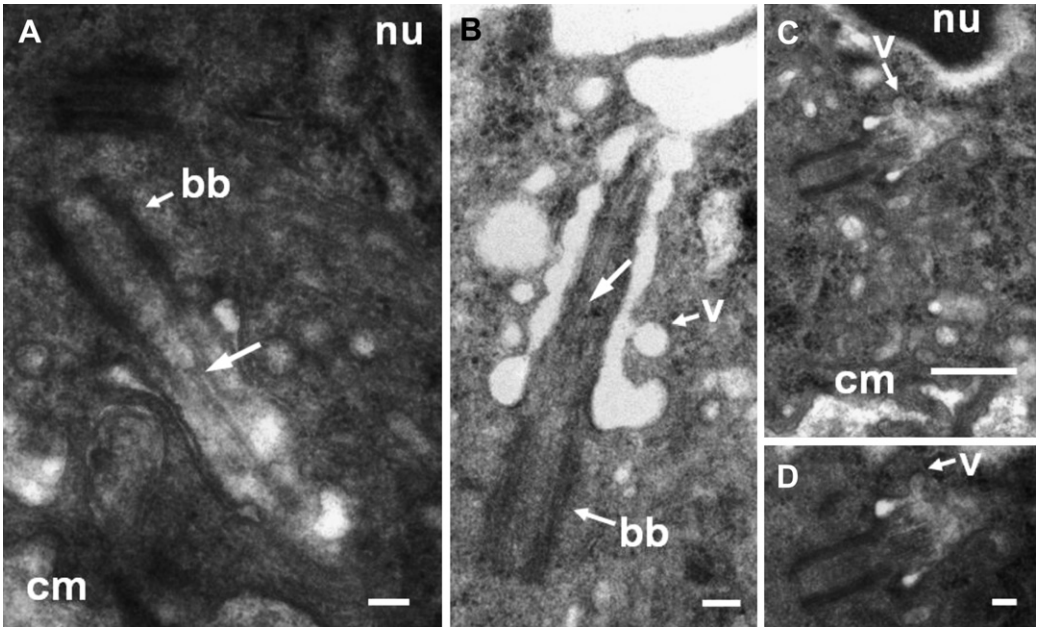

Fig. 2. Transmission electron micrographs of chondrocyte primary cilia in intact femoral cartilage. (A, B) Sections transecting the longitudinal axis of the basal body (bb) typically display a short portion of the ciliary axoneme (arrow) before the axoneme deflects out of the plain of the section. (B–D) Chondrocyte primary cilium are typically positioned within a membranous invagination into the cell and adjacent to the nucleus (nu) rather than at the cell margin (cm) as seen in other cell types. Endocytotic vesicles (v) are also found along the membrane lining the invagination housing the ciliary axoneme (Fig. D higher magnification of C). Scale bar 100 nm (A, B, D) or 500 nm (C). Images taken at 30,000× (A), 15,000× (B), 12,000× (C), or 25,000× (D).

1, LAMP-1 and CD44 - the chondrocyte receptor responsible for mediating endocytosis of hyaluronan[25], and their proximity to the chondrocyte ciliary pocket (Fig. 6). EEA-1 and LAMP-1 was visualised as intensely stained punctuate dots across the chondrocyte membrane [Fig. 6(B and E)]. In contrast, CD44 stained in larger patches across the chondrocyte membrane [Fig. (6H)]. Importantly, we found EEA-1 and CD44 to colocalise with acetylated α-tubulin, and likely located towards the entrance of the ciliary pocket [Fig. 6(B,C,H,I)].

Discussion

In this study, we have imaged live chondrocyte primary cilia across different regions of intact murine femoral condyles and after osmotic challenge for the first time. This microscopy technique has been used successfully to image primary cilia in tenocytes[26,27], but not chondrocyte primary cilia *ex vivo*. This technique negates disruption of the cartilage/bone interface and chemical fixation that are required when harvesting and processing explants or primary chondrocyte cultures. The utilization of live chondrocytes in their undisturbed extracellular matrix allowed us to quantify changes to primary cilia length in response to hypo- and hyper-osmotic challenge within minutes of its application and to map primary cilia incidence, length and orientation across different regions of the same cartilage surface.

Chondrocyte primary cilia length decreased significantly after a one minute hypo- or hyper-osmotic challenge while primary cilia incidence remained unchanged. In our length measurements we did not distinguish between the intracellular and extracellular length of the primary cilium and therefore this decrease is in the length of the axoneme as opposed to a retraction of the primary cilium into the ciliary pocket. It is possible that the shortening of primary cilia length is a resorbtion of the primary cilia by the chondrocyte in response to moving through the cell cycle, and not due to the osmotic challenge[28,29]. This is unlikely because (1) the femora used in this study were from adult mice where the majority of the chondrocytes would presumably be in the G_0 phase of the cell cycle, and (2) we would expect the decrease in primary cilia length to be accompanied by a decrease in primary cilia incidence which was not the case. Therefore the change in length measured within minutes of the application of osmotic challenge is most likely a response to osmotic loading. During prolonged periods (>24 hours) of mechanical loading primary cilia length and incidence decreases on chondrocytes seeded in agarose[14]. Our results show that chondrocyte primary cilia shorten in response to osmotic challenge as well as compressive loads and do so sooner (minutes) after the environmental change.

It has been shown that primary cilia length and incidence are both increased in osteoarthritic cartilage[19] where the chondrocytes are presumably exposed to a chronic (months or years) hypo-osmotic environment in glycosaminoglycan depleted extracellular matrix. These data together with the present study may suggest differential responses of chondrocyte primary cilia to acute (primary cilia shorten) and chronic (primary cilia lengthen) exposure to a hypo-osmotic challenge. It is also important to note that *in vitro* microtubule assembly occurs in a matter of seconds in response to various stimuli[30,31], although ciliary microtubules are somewhat more stable than other cytoplasmic microtubules due to acetylation and detyrosination. While there will presumably be a delayed response *ex vivo* as the stimulus reaches the chondrocytes, it is possible that primary cilia shortening may occur much faster than measured in the present study. It will be important in the future to measure primary cilia incidence and length after longer (hours) and shorter (seconds) exposure of live *ex vivo* chondrocytes to osmotic challenge.

In addition to osmotic effects, we also report that primary cilia length varied between condyles and across the surface of each condyle. Primary cilia in the inside and outside regions were longer on the medial than the lateral condyle and primary cilia were longest in the inside compared to all other regions of the medial condyle. The values of live chondrocyte primary cilia length reported here are consistently shorter than those previously

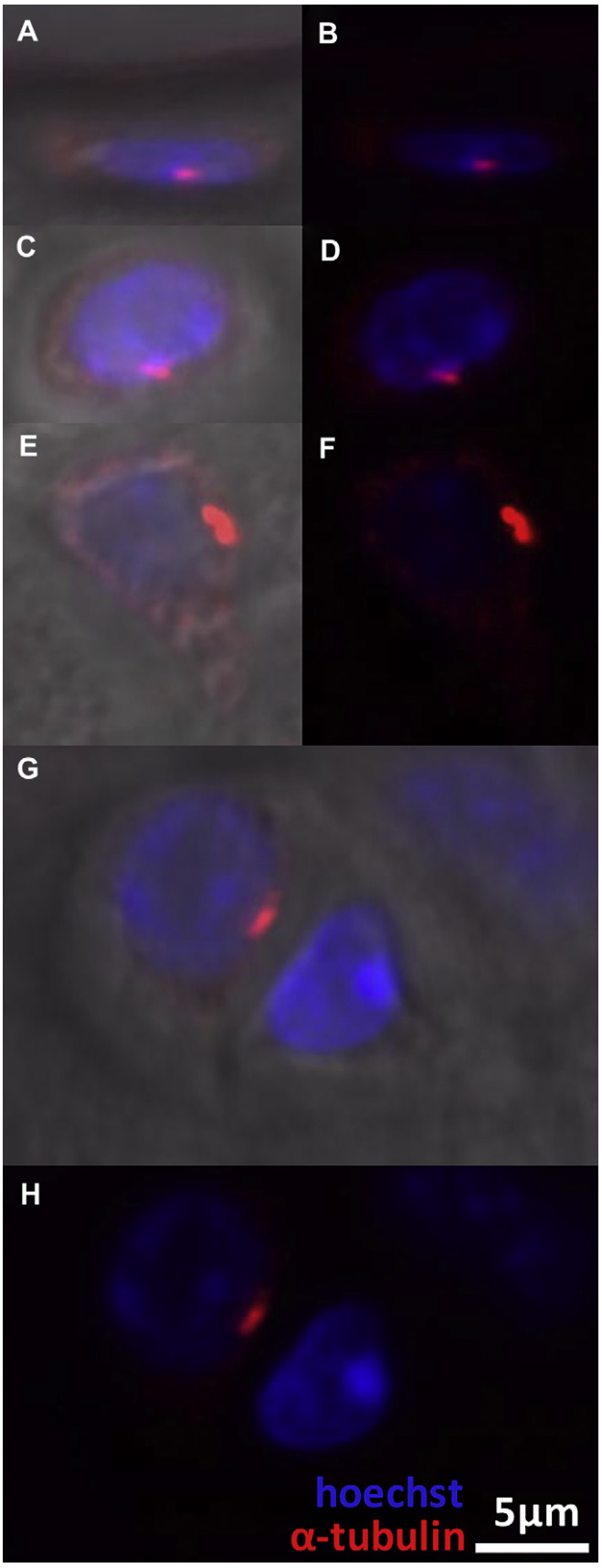

Fig. 3. Immunocytochemistry images of single chondrocytes from the superficial zone (A, B), middle zone (C, D) and calcified cartilage region along the cartilage/bone interface (E, F) or two chondrocytes sharing a chondron (G, H). Images show DIC images overlayed with fluorescence (A, C, E, G) or fluorescence alone (B, D, F, H) with acetylated α-tubulin labeled in red and nuclei in blue. Primary cilia are seen as a bright red streak adjacent to the nucleus with the majority of its shaft invaginated in a membranous pocket in the chondrocyte and not protruding into the surrounding extracellular matrix. Chondrocytes have a single primary cilia located on the basal side of individual chondrocytes in the superficial and middle cartilage zones (A–D), on either of the two sides perpendicular to the articular surface in the cartilage/bone interface region (E, F) and on the side adjacent to the second cell within a chondron (G, H).

reported (Table I). It is important to note that the primary cilia length values reported in this study are likely an underestimation of total primary cilia length. This underestimation is due to the primary cilium not being aligned in the imaging plane. Furthermore, we were unable to control for inclusion of the basal body in our length measurements due to our live cell imaging technique. Because our methods were consistent across specimens and experimental manipulations however, we believe that differences in primary cilia length are the result of actual biological variation.

The percentage of live cells with primary cilia reported in this study (87%) compares well with the early *in situ* work of Wilsman *et al.*[6,18,32] but is larger than that reported in cultured cells in agarose[14,20] (Table I). This discrepancy is perhaps due to chondrocytes moving out of the G_0 and G_1 phases of the cell cycle during isolation and culture[28,29]. In the present study we measured primary cilia incidence across different regions of the same cartilage surface and compared left and right femora for the first time. We found primary cilia incidence to be consistent across the femoral condyles but larger on the right compared to the left femur. The biological significance of this difference is unclear. Handedness does occur in mice but with equal incidence on either side[34], therefore our result is unlikely to imply a difference in the use of left and right legs. These data together would suggest that primary cilia incidence is significantly reduced after chondrocyte isolation and varies between the cartilages of different bones but not across the same cartilage surface.

By utilising immunocytochemistry and *ex vivo* live cell imaging we were able to evaluate primary cilia orientation in both the saggital and coronal planes. In the saggital plane, we found primary cilia positioned on the inferior aspect of the cell relative to the articular surface throughout cartilage depth and oriented between cells within a chondron. Close to the calcified cartilage region, primary cilia were found on the sides of the chondrocytes perpendicular to the subchondral bone. These findings are similar to those of McGlashan *et al.*, 2008 except for their report of primary cilia on the 'vertical' sides of chondrocytes in the middle and deep zones of the cartilage[19]. This discrepancy in the findings of our two studies is most likely due to the contrast (two orders of magnitude) in thickness between bovine cartilage used in the work of McGlashan *et al.*, 2008[19] and our mouse cartilage[35] and thus the thickness and distinction of zones throughout cartilage depth. We propose that the positioning of the primary cilia on the basal side of the chondrocyte is likely an effort to protect it and the neighbouring golgi apparatus from tensile and compressive forces sustained by chondrocytes during joint loading[19] or perhaps critical for appositional sequestration of extracellular matrix molecules.

In the coronal plane, we were surprised to find that primary cilia orientation did not vary across different sites of the femoral condyle. Our data is in agreement with Farnum and Wilsman (2011) who recently reported an essentially random orientation of chondrocyte cilia from load bearing and non load bearing regions of the equine femoral condyles in the coronal plane[36]. It would seem that chondrocyte primary cilia orientation is influenced to a greater extent by the microenvironment created by the encapsulating pericellular matrix (lying parallel to the chondrocyte membrane)[37] than the properties of the extracellular cartilage matrix surrounding them.

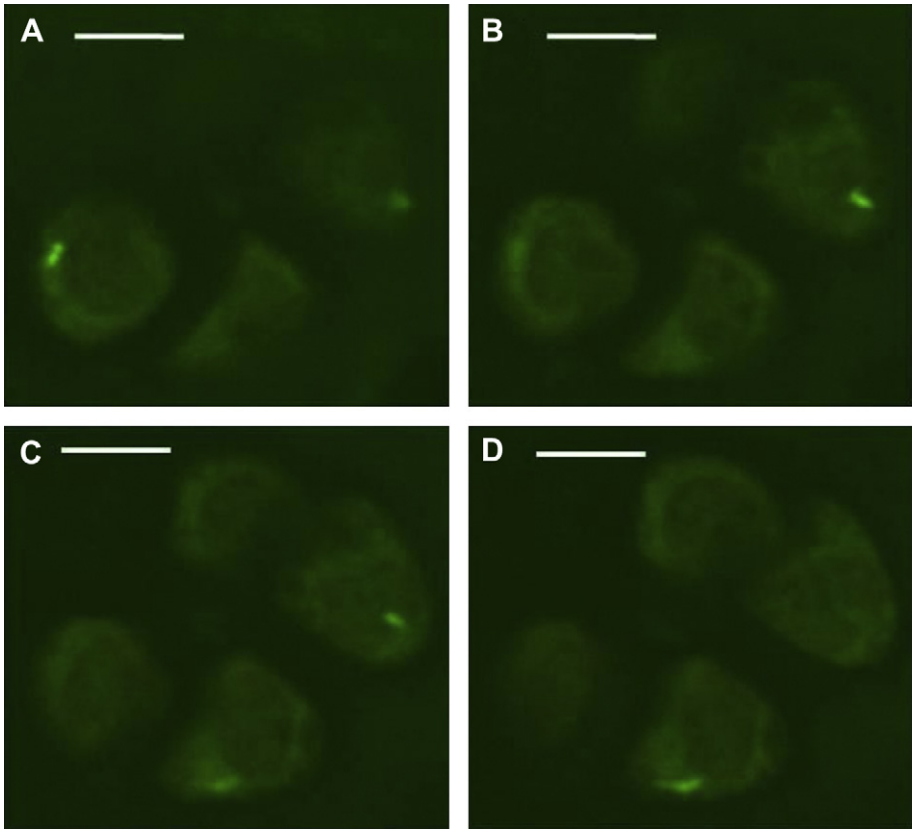

Fig. 4. A vertical confocal stack through four live chondrocytes showing the primary cilia as bright green streaks against the more faintly stained tubulin throughout the cytoplasm. Note how each cell has a single primary cilium with one end close to the nucleus (darker circle within the chondrocyte) with its shaft extending within a membranous invagination through the cytoplasm and the other end close to the extracellular matrix. Scale bar 10 μm.

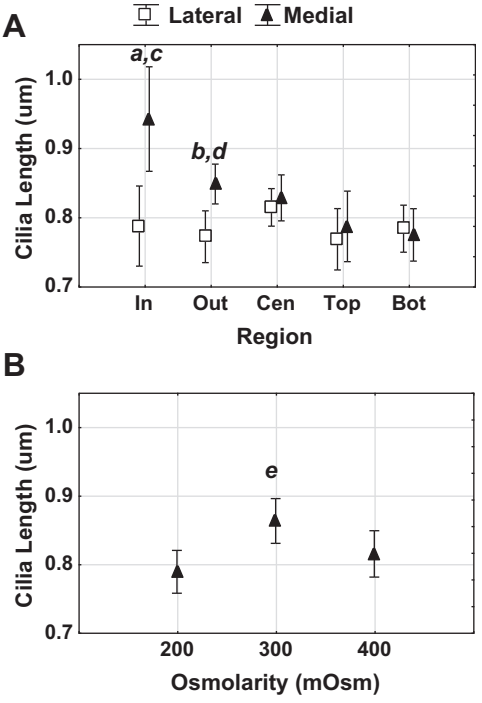

Fig. 5. Graphs showing primary cilia length (μm) as a function of (A) condyle (medial/lateral) and region (inside (In), outside (Out), centre (Cen), top (Top) and bottom (Bot) and (B) osmolarity (mOsm)). Statistically significance differences are indicated from (a) lateral inside, $P < 0.001$, (b) lateral outside, $P = 0.002$, (c) medial outside $P = 0.019$, (d) medial bottom $P = 0.002$ and (e) 200 and 400 mOsm ($P = 0.002$ and $P = 0.045$ respectively). $N = 4$ for each mean and error bars represent 95% confidence intervals.

Thus, in the coronal plane, chondrocyte primary cilia appear to be randomly orientated.

Much attention and thought has been given to the primary cilia projection and its potential to act as a highly sensitive probe capable of detecting changes in the extracellular matrix environment and enabling the chondrocyte to respond rapidly through the close proximity of the primary cilia base to the golgi apparatus and nucleus[33]. The localisation of a range of matrix receptors[11], connexin 43 hemichannels[12] and TRPV4[13], in addition to our observation that primary cilia are capable of shortening within minutes of osmotic challenge would support such a hypothesis. Most recently, however, attention has been turned to the role of the primary cilia shaft that is invaginated in the ciliary pocket close to the nucleus[7–10]. The ciliary shaft has been shown to be enclosed by a 'ciliary pocket' lined with endocytotic sites with the potential for control of the pocket volume *via* dynamic changes in actin fibre bundles[7,8]. Together (transmission electron microscopy (TEM), immunocytochemistry and live cell confocal) our data are in agreement that the majority of the length of the chondrocyte primary cilia is invaginated in a ciliary pocket close to the nucleus as opposed to projecting out from the chondrocyte into the surrounding matrix. We have shown some evidence for clathrin coated endocytotic vesicles surrounding the cilia pocket in the chondrocyte cytoplasm and colocalization of endosomal proteins and CD44 – the receptor for hyaluronan that is endocytosed by chondrocytes[25] – with the entrance of the primary ciliary pocket. CD44 was previously reported to be excluded from the chondrocyte primary cilia pocket[11] however this study used a monoclonal antibody to the clone OS/37 as the primary antibody to CD44 unlike the polyclonal antibody to the synthetic peptide surrounding amino acid 39 used in the present study. Together these data

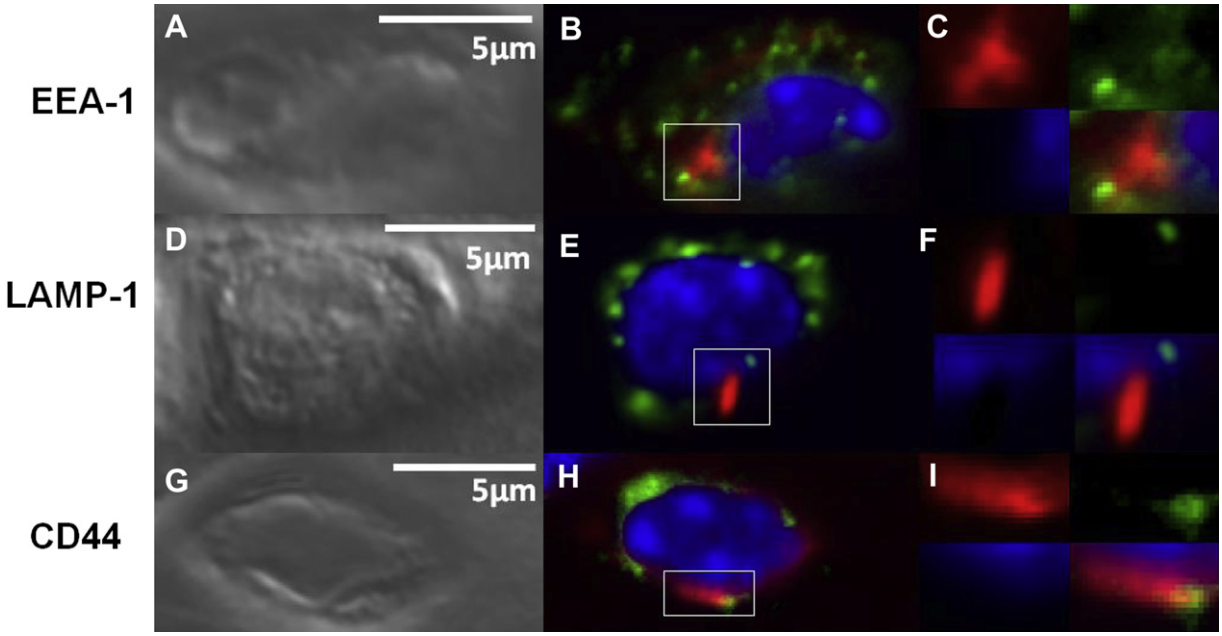

Fig. 6. Immunocytochemistry images of single chondrocytes from the middle zone (A–C, G–I) or the deep zone (D–F). Images are DIC (A, D, G) or fluorescence (B, E, H) at the same scale with acetylated α-tubulin labeled in red, nuclei in blue and EEA-1 (B), LAMP-1 (E) or CD44 (H) in green. The boxed areas in B, E and H are shown at a higher magnification in C, F and I with the red, green and blue fluorescence shown separately and also overlayed. Note the colocalization (orange/yellow colour) of EEA-1 and CD44 likely at the entrance to the ciliary pocket (C, I).

Table I
Summary of primary cilia incidence and length measurements from the literature

Author	Species	Age	Preparation	Site	Incidence (%)	Length (μm)
Wilsman, 1978[32]	Canine	Adult	Explant	Lateral femoral condyle	93.6	N/A
Wilsman and Fletcher, 1978[6]	Canine	Infant	Explant	Humeral head	100	1.76
Wilsman *et al.*, 1980[18]	Equine	Adult	Explant	Lateral femoral condyle and femoral trochlea	96	N/A
Wilsman *et al.*, 1980[18]	Murine	Adult	Explant	Lateral femoral condyle	100	N/A
Poole *et al.*, 2001[33]	Galline	Embryo	Explant	Sterna	100	1–4
McGlashan *et al.*, 2008[19]	Bovine	Adult	Explant	Patellae	46	1.1–1.5
Kaushik *et al.*, 2009[20]	Murine	Adult	Agarose culture	Knee joint	26–28	N/A
McGalashan *et al.*, 2010[14]	Bovine	Adult	3D agarose construct	Metacarpo-phalangeal joint	15–45	1.3–2.2
Current study	Murine	Adult	*ex vivo*	Femoral condyle	87	0.75–0.95

suggest a significant role for the invaginated length of chondrocyte primary cilia in addition to, and perhaps equally important to, that portion projected into the extracellular matrix.

In conclusion we present measurements of live *ex vivo* chondrocyte primary cilia and demonstrate that they are capable of shortening within minutes in response to osmotic challenge. Furthermore, we provide subcellular and cellular evidence that chondrocyte primary cilia are deeply invaginated in a ciliary pocket which contains sites for endocytosis.

Author contributions

A.L. Clark contributed to the conception and design of this study, analysis and interpretation of the data and drafting, critical reviewing and final approval of the article.

D.R. Rich contributed to the design of this study, collection, analysis and interpretation of the data and drafting, critical reviewing and final approval of the article.

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Conflict of interest

No author has any competing interests.

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